Purification and Preliminary Characterization of Stratum Corneum Chymotryptic Enzyme: A Proteinase That May Be Involved in Desquamation

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In recent work we have shown that a serine proteinase, stratum corneum chymotryptic enzyme, with properties compatible with a role in desquamation *in vitro* as well as *in vivo*, is generally present in human stratum corneum. The enzymologic properties of the stratum corneum chymotryptic enzyme in a KCl extract of dissociated plantar corneocytes were compared with those of other known chymotryptic serine proteinases. Stratum corneum chymotryptic enzyme was found to differ significantly from bovine chymotrypsin, human cathepsin G, and human mast cell chymases in regard to inhibitor profile and substrate specificity. Stratum corneum chymotryptic enzyme was further purified from KCl extracts of dissociated plantar corneocytes by affinity chromatography on gels with covalently linked soybean trypsin inhibitor. The purified preparation contained one major component with apparent molecular weight 25 kD and one minor component with slightly higher apparent molecular weight as revealed by Coomassie staining after electrophoresis in polyacrylamide gels with sodium dodecyl sulphate of samples that had not been reduced. Both these components were associated with chymotrypsinlike activity as revealed by zymography in polyacrylamide gels with co-polymerized casein. On zymography gels, the purified preparation was also found to contain minor amounts of components with trypsinlike activity. The major purified protein had an apparent molecular weight of around 28 kD after reduction and full denaturation and was shown to contain carbohydrate.
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A strictly controlled balance between *de novo* production of corneocytes and cell shedding from the skin surface in the process of desquamation is fundamental for epidermal homeostasis. Although the details of the desquamation process still remain to be elucidated fully, some new insights have been obtained recently.

In studies on an *in vitro* system [1–3], in which a desquamation-like process from plantar stratum corneum takes place, evidence has been obtained that the cohesion between individual corneocytes is mediated to a significant extent by protein structures, including desmosomes, and hence that proteolytic mechanisms may play a crucial role in the cell-shedding process. From these experiments some basic properties of the involved proteinase(s) could be deduced. Evidence of the importance of proteolytic processes in desquamation also in non-palmoplantar stratum corneum has also been presented [4]. To fully understand the mechanisms of desquamation, a detailed knowledge about the proteolytic enzyme(s) involved is needed.

We recently reported on a new chymotrypsin-like proteinase, stratum corneum chymotryptic enzyme (SCCE), present in palmoplantar [5] as well as non-palmoplantar [6] stratum corneum. This enzyme has an inhibitor profile similar to the cell shedding and the degradation of the desmosomal protein desmoglein I in plantar stratum corneum *in vitro*. It is most likely extracellularly located in the stratum corneum [7], and has a pH dependency allowing it to be active in the stratum corneum under physiologic conditions [5,6]. Thus, SCCE has properties compatible with a function in physiologic desquamation.

This report presents a further characterization of SCCE. A comparison between SCCE and some other well-known chymotryptic enzymes regarding the effects of inhibitors and substrate specificity has been carried out. The enzyme has also been purified, and some of its molecular properties are presented.

MATERIALS AND METHODS

Aprotinin, chymostatin, leupeptin, Suc-Ala-Ala-Pro-Phe-pNA, bovine chymotrypsin (A_4), soybean trypsin inhibitor (SBTI), and Glycan Detection Kit were purchased from Boehringer, Mannheim, Germany. Cathepsin G from purulent human sputum was from E. Lotti, Geneva, Switzerland. MeO-Suc-Arg-Pro-Tyr-pNA (S-2586) and H-D-IIe-Pro-Arg-pNA (S-2288) were obtained from Kabi Diagnostica, Mölndal, Sweden. Affigel 15 was from BioRad, Richmond, California.

Hyperplastic human plantar stratum corneum was collected through cooperation with the Society for Swedish Pedicyrists. Only material obtained by means of clippings or cuttings was used. Material was not collected from feet with scaling disorders. Before being mailed, the stratum corneum was air dried and packeted in plastic bags. In the laboratory it was stored at −20°C until used.

Proteinase Assays with Chromogenic Substrates Incubations were performed at 37°C in microtiter plates. The total incubation volume was 125 μl (comparison of activities toward various chymotrypsin substrates and assays of chromatograms) or 135 μl (experiments with inhibitors). Each incubation mixture contained Tris-HCl pH 8.0 (final concentration 0.08 M), KCl (final concentration 0.2 M), 100 μl substrate solution, 25 μl enzyme source (appropriately diluted in 0.1 M Tris-HCl pH 8.0, 1 M KCl),

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Abbreviations: S-2288, H-D-IIe-Pro-Arg-pNA; S-2586, MeO-Suc-Arg-Pro-Tyr-pNA; SBTI, soybean trypsin inhibitor; SCCE, stratum corneum chymotryptic enzyme.

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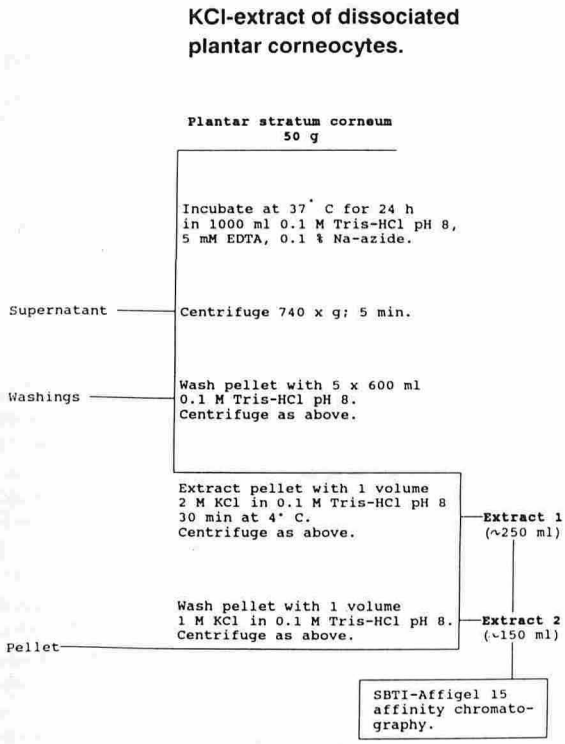

Figure 1. Schematic outline of the preparation of SCCE-containing KCl extracts of dissociated plantar corneocytes. For each subsequent affinity chromatography step, extracts 1 and 2 from two preparations from 50 g each of plantar stratum corneum were pooled.

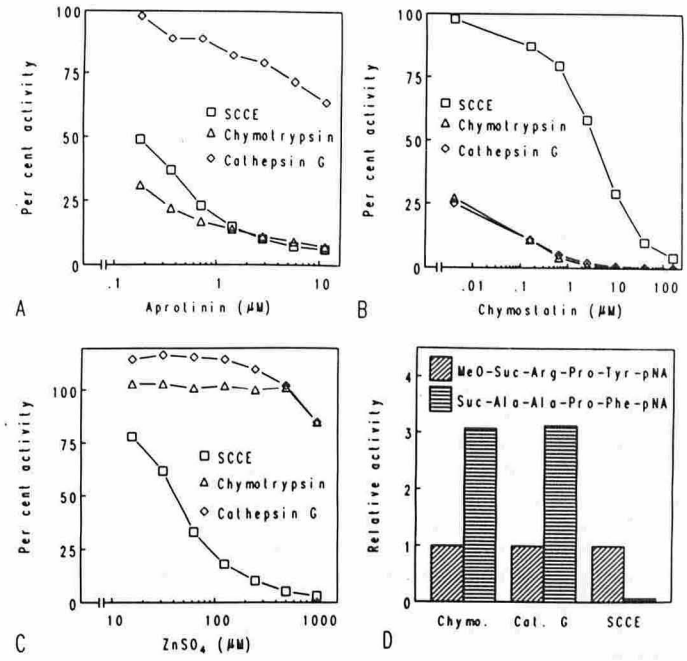

Figure 2. Comparison of SCCE, bovine chymotrypsin, and human cathepsin G regarding the effects of inhibitors (*A*, aprotinin; *B*, chymostatin; *C*, zinc sulphate), and *D* substrate specificity. *A–C*, MeO-Suc-Arg-Pro-Tyr-pNA (S-2586, initial concentration 1.2 mM) was used as substrate. The enzyme activity with no inhibitor present was standardized to 100%. *D*, The initial concentration of both substrates was 1.2 mM. The enzyme activity with MeO-Suc-Arg-Pro-Tyr-pNA was standardized to one arbitrary unit. Source of SCCE = KCl extract (extract 1 in Fig 1) of dissociated plantar corneocytes.

and eventually 10 μl inhibitor solution. Stock solutions of S-2586 and S-2288 were prepared in distilled water, Suc-Ala-Ala-Pro-Phe-pNA in 1-methyl-2-pyrrolidone (final concentration of solvent in incubation mixtures 4%), and chymostatin in dimethyl sulphoxide (final concentration of solvent in incubation mixtures 1%). Further compositional data are given in legends to figures. At the end of the incubations (1.5–3 h) 125 μl of 10% acetic acid was added to each well and the absorbance read at 405 nm with incubation mixtures without added enzymes as blanks. The amounts of added enzymes were adjusted to give a change in absorbance at 405 nm at the end of the incubations of 0.3–0.7.

Electrophoresis Electrophoresis in polyacrylamide gels with sodium dodecyl sulphate (SDS-PAGE) was carried out according to Laemmli [8] in 12.5% gels. Aliquots of fractions from affinity chromatography, in some instances concentrated approximately 20 times by centrifugal filtration with Ultrafree MC filters (cut off 10 kD; Millipore, Bedford, MA), were mixed with an equal volume of sample buffer with or without dithiothreitol (50 mM). Only samples to be reduced were heated on a boiling water bath for 2 min before application on the electrophoresis gels. For analytical gels (thickness 0.75 mm) 5-mm slots were used. Protein bands were visualized with Coomassie Blue.

Casein Hydrolyzing Activity The procedure of Horie *et al* [9] was adopted for the separation of proteinases by electrophoresis of unreduced samples in polyacrylamide gels with SDS and co-polymerized casein. Gels (12.5%) were prepared and run according to Laemmli [8], but included 0.1% heat-denatured casein in the polymerization mixture. After electrophoresis, the gels were soaked for 1 h at room temperature in 2.5% Triton X-100 in 0.1 M Tris-HCl, pH 8.0, and then incubated at 37°C for 3 h in 0.1 M Tris-HCl pH 8.0. Gels were in some experiments incubated in the presence of inhibitors, which were then also present in the Triton X-100 solution [5]. Casein-degrading activity could be visualized as clear bands when the gels were stained with Coomassie Blue.

Preparation of SBTI-Affigel 15 Fifty milligrams of SBTI was coupled to 12 ml sedimented gel according to the recommendations of the manufacturer. Remaining active groups of the gel were blocked with ethanolamine.

Detection of Carbohydrates Carbohydrates were detected using the Glycan Detection Kit according to the recommendations of the manufacturer. SDS was added to aliquotes of fractions from affinity chromatography with $A_{280\ nm}$ about 0.2% to a final concentration of 0.1%. The fractions were then dialyzed against 0.1 M acetate buffer, pH 5.5, 0.1% SDS, reacted with sodium metaperiodate and digoxigenin-succinyl-amidocaproic acid hydrazide, and then concentrated approximately four times in dialysis tubings immersed in ficoll (Pharmacia LKB, Sollentuna, Sweden). After SDS-PAGE (no reduction of samples) and electrophoretic transfer to nitrocellulose membranes digoxigenin-containing protein bands were detected with a sheep anti-digoxigenin serum conjugated with alkaline phosphatase [10]. Bovine transferrin, 0.1 mg/ml, served as positive control; samples prepared as above but with no periodate oxidation served as negative controls.

RESULTS

Comparison Between SCCE, Bovine Chymotrypsin, and Human Cathepsin G In these experiments the source of SCCE was KCl extracts of dissociated plantar corneocytes prepared as in Fig 1 and further described below. The results are summarized in

Table I. Summary of Preparation of KCl Extracts of Dissociated Plantar Corneocytes (also see Fig 1)[a]

	Volume (ml)	$A_{280\ nm}$	S-2586 Activity[b]	S-2288 Activity[b]
Tris-EDTA supernatant	750	56.0	156	421
Washing				
1	600	17.0	204	150
2	600	4.4	151	90
3	600	2.4	63	45
4	600	1.4	58	35
5	600	1.0	29	15
Extract 1 (2 M KCl)	240	1.9	993	131
Extract 2 (1 M KCl)	140	1.1	280	59

[a] Starting material: 50 g dried plantar stratum corneum.
[b] Total: $\Delta A_{405\ nm} \times h^{-1}$.

Table II. Summary of SBTI Affigel 15 Affinity Chromatography of SCCE

	Volume (ml)	A_{280} nm × ml	S-2586 Activity[a]		S-2288 Activity[a]	
			Total	Percent	Total	Percent
KCL extract (pooled from 100 g plantar stratum corneum)	700	1290	2534	100	384	100
Breakthrough fraction	700	1290	327	13	320	83
Eluate 1 mM HCl	14	0.56	4	0.16	0	0
Eluate 10 mM HCl	14	0.64	1054	42	31	8
Eluate 100 mM HCl	17.5	0.18	533	21	8	2

[a] $\Delta A_{405\ nm} \times h^{-1}$.

Fig 2. For the studies on the effects of inhibitors, S-2586 was used as substrate. The efficiency of aprotinin as an inhibitor of SCCE and chymotrypsin was high and approximately the same for the two enzymes. The effect of cathepsin G, on the other hand, was much less (Fig 2*A*). Chymostatin caused inhibition of all three enzymes, but the inhibitor concentration that caused 50% inhibition was more than three orders of magnitude higher for SCCE than for chymotrypsin and cathepsin G (Fig 2*B*). Zinc sulphate was an efficient inhibitor of SCCE, but not of chymotrypsin and cathepsin G (Fig 2*C*). The activity of the three enzymes against the substrates S-2586 and Suc-Ala-Ala-Pro-Phe-pNA are compared in Fig 2*D*. Because the object was to find similarities or differences between the enzymes examined, these experiments were carried out at only one initial concentration for each substrate. Whereas Suc-Ala-Ala-Pro-Phe-pNA appeared to be a significantly better substrate than S-2586 for chymotrypsin and cathepsin G, the reverse was found for SCCE.

Purification of SCCE As a first purification step, KCl extracts of dissociated plantar corneocytes were prepared. The procedure, which results in a considerable purification of SCCE in regard to total protein, was based on recent findings described by Egelrud and Lundström [5] and is summarized in Fig 1 and Table I. Cell dissociation in plantar stratum corneum was induced by means of incubating the tissue at pH 8 in the presence of ethylenediamine tetraacetic acid (EDTA) at 37°C [1]. SCCE is found in association with the dissociated corneocytes at low ionic strength, but is solubilized as the ionic strength is increased [5,7]. The dissociated cells were washed and then extracted with KCl without homogenization. The cell dissociation was not complete after 24 h of incubation, i.e., the pellet on which Fig 1 is based includes fully dissociated cells as well as fragments of still cohesive tissue. The yield of SCCE-related activity did not increase if the incubation at 37°C was prolonged to more than 24 h. In addition to enzyme activity toward S-2586 (chymotrypsin substrate), the KCl extracts contained some activity toward S-2288 (a substrate for a wide range of serine proteinases with trypsin-like substrate specificity).

Further purification of SCCE was obtained by affinity chromatography on SBTI Affigel 15; the results are summarized in Table II and Figs 3, 4, and 5*B*. Whereas approximately 90% of the S-2586–hydrolyzing activity was bound to the affinity gel, only about 20% of the S-2288–hydrolyzing activity was bound. Both activities could be eluted from the gel with 10 to 100 mM HCl. The total yield of S-2586–hydrolyzing activity (breakthrough fraction plus eluates) was around 70%; 63% was recovered in the HCl eluates. The total amount of protein in fractions containing enzyme activity was about 0.5 to 0.8 mg (assuming an absorptivity at 280 nm of $1/mg \times ml^{-1}$).

Molecular Weight Analysis The results of SDS-PAGE analysis of unreduced, concentrated samples of fractions of the eluate are shown in Fig 4*A*. Fractions with enzyme activity contained one major band, apparent molecular weight about 25 kD, that accounted for more than 90% of the Coomassie-positive material, and one minor component with slightly higher apparent molecular weight. Zymography in SDS-PAGE gels containing casein (Fig 4*B*) revealed one major and one minor band with caseinolytic activity and with the same electrophoretic mobility as the Coomassie-positive components. These two caseinolytic components could be inhibited by chymostatin but not by leupeptin. In addition, the fractions contained minor proteolytic components with apparent molecular weights of approximately 30 kD, which were not inhibited by chymostatin but could be inhibited by leupeptin (results of experiments with inhibitors in zymography gels not shown).

Reduction and heat denaturation before electrophoresis resulted in an apparently higher molecular weight (≈28 kD) for the major Coomassie-positive component (Fig 5*A, lane 2*), as compared with unreduced samples (Fig 5*A, lane 1*). Also, after reduction, a minor component with slightly higher electrophoretic mobility than the major component was found. In addition, reduction revealed one minor component on overloaded gels (not shown) with apparent molecular weight of about 22 kD and Coomassie-positive material that moved with the front on electrophoresis. The 22-kD component and the low molecular weight material may represent products of autodigestion because they tend to increase in amount in samples stored at room temperature. In Fig 5*B* is shown a comparison of the SDS-PAGE pattern for the proteins of the starting material for affinity chromatography (KCl extract of dissociated plantar corneocytes) and the purified SCCE.

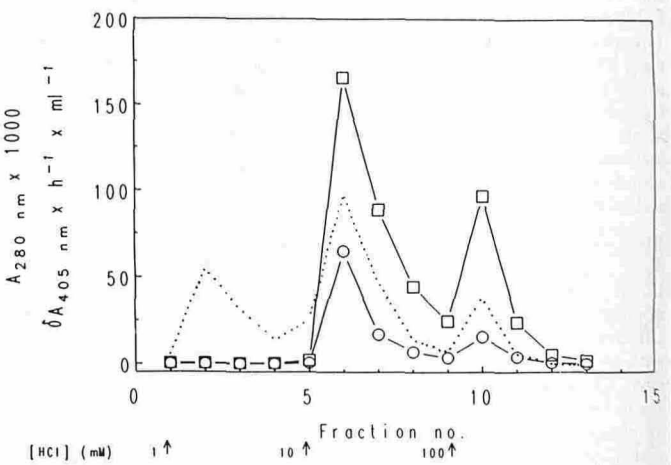

Figure 3. Affinity chromatography of SCCE on SBTI Affigel 15. Combined KCl extracts prepared from 100 g (dry weight) of plantar stratum corneum (total volume 700) were filtered through filter paper and run through a 0.8 × 2 cm bed of SBTI Affigel 15 packed in a glass column, flow rate 42 ml/h. The column was washed with 0.1 M Tris-HCl, pH 8.0, 1 M KCl until the absorbance of the eluate was below 0.01 and then with 10 ml of 0.1 M Tris-HCl pH 8.0 to remove the KCl. Stepwise elution of bound material was carried out with increasing concentrations of HCl as indicated. The eluent was changed when the absorbance of the eluate had decreased to below 0.01. Three-milliliter fractions were collected in test tubes containing Tris-HCl, pH 8, total volume 0.4 ml, in an amount calculated to be enough to adjust the pH of the eluate to 7. The pH of each fraction was immediately checked and, if necessary, adjusted to 7 with small volumes of 1 M Tris-HCl, pH 8. Protein concentration was estimated by measurement of absorbance at 280 nm. *Dotted line,* absorbance at 280 nm; *solid line with open squares,* activity toward S-2586; *solid line with open circles,* activity toward S-2288 multiplied 10 times. The initial concentration of both substrates in the assay mixtures was 1.1 mM.

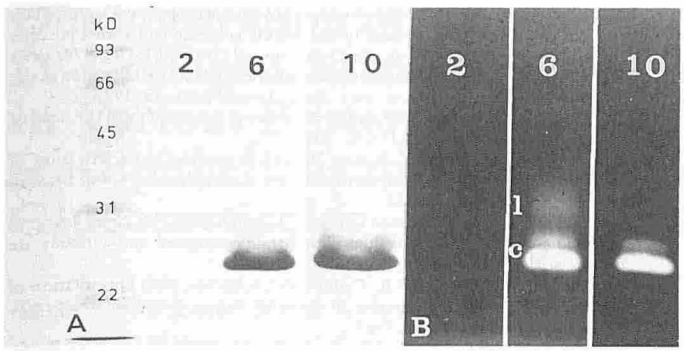

Figure 4. SDS-PAGE and Coomassie staining *(A)*, and zymography after SDS-PAGE with co-polymerized casein *(B)* of fractions 2, 6, and 10 from the chromatogram shown in Fig 3. Gels 12.5%, unreduced samples. Before being prepared for electrophoresis, the samples had been concentrated about 20 times in *A*, and diluted 10 times in *B*. Molecular weight markers to the left. *B*, 1, group of caseinolytic components that could be inhibited by leupeptin (160 μM); c, group of caseinolytic components that could be inhibited by chymostatin (40 μM).

Carbohydrate Content After periodate oxidation and coupling with digoxigenin, two components with the same electrophoretic mobility as the two Coomassie-positive bands that could be detected after SDS-PAGE, and which comigrated with chymostatin-inhibited casein-hydrolyzing enzyme activity, reacted with the antidigoxigenin serum. When the periodate oxidation was omitted, no components could be detected (results not shown). Thus SCCE appears to contain carbohydrate.

DISCUSSION

KCl extracts of dissociated plantar corneocytes have recently been shown to contain one major caseinolytic proteinase with apparent molecular weight of about 25 kD in unreduced form that comigrates with S-2586-hydrolyzing activity on gel exclusion chromatography [5]. This enzyme, in addition to being active toward a chymotrypsin substrate, was inhibited by chymostatin, but not by leupeptin; hence, the proposed name "stratum corneum chymotryptic enzyme." This enzyme was inhibited also by phenylmethylsulphonyl fluoride, aprotinin, and SBTI [5], which suggests that it is a serine proteinase. Because SCCE, as well as unipolar cell shedding from plantar stratum corneum *in vitro* [3] and the associated degradation of the desmosomal glycoprotein desmoglein I [2] were all inhibited by aprotinin, chymostatin, and zinc ions, and because SCCE is active at pH 5.5 in spite of a pH optimum of about 7–8 [5], it was concluded that SCCE may play a role in physiologic desquamation. This is supported also by the fact that an enzyme with the same properties as SCCE is present also in non-palmoplantar stratum corneum [6], and that SCCE appears to be active in the stratum corneum intercellular space [7].

Results given in the present work suggest that SCCE differs enzymologically from other chymotryptic proteinases. The inhibitor profile and the ability to degrade two different substrates of chymotrypsinlike enzymes were significantly different for SCCE as compared with bovine chymotrypsin and human cathepsin G. SCCE also seems to differ from human mast cell chymase. The latter enzyme catalyzes the degradation of Suc-Ala-Ala-Pro-Phe-pNA efficiently [11,12], which SCCE does not, and is not inhibited by aprotinin or SBTI [13,14].

There is evidence that the major Coomassie-positive component purified by affinity chromatography in this work is identical with SCCE. The fact that it could be bound to and eluted from insolubilized SBTI suggests that it is a proteinase. Under non-reducing conditions, it had the same electrophoretic mobility as the major caseinolytic enzyme in the eluate from the affinity column as well as in KCl or SDS extracts of dissociated plantar corneocytes [5]. This caseinolytic enzyme, which could be inhibited by chymostatin but not by leupeptin, was co-eluted from the affinity gel with activity toward S-2586. The minor Coomassie-positive component, with slightly higher apparent molecular weight, co-chromatographed with the major component on SBTI Affigel 15, and had the same electrophoretic mobility under non-reducing conditions as a minor caseinolytic component that was also inhibited by chymostatin. At present, the relationship between the two components is unclear. The eluate from the affinity column also contained enzyme activity toward S-2288. It seems reasonable to assume that this activity corresponds to the minor caseinolytic components with apparent molecular weights around 30 kD that could be found in the peak fractions and could be inhibited by leupeptin but not by chymostatin. No Coomassie-positive components of this size could be detected, which suggests that enzymes with trypsinlike activity were also bound to and eluted from the SBTI affinity column, but in minor amounts only, as compared with enzymes with chymotryptic activity, most of which may be SCCE.

Recent studies [7] have shown that SCCE is associated with plantar corneocytes in a way that allows it to be catalytically active in the extracellular space. That SCCE is secreted to the extracellular space (or is a membrane-associated enzyme with a part of the molecule exposed on the cell surface), is supported also by findings in the present study, where it is shown that SCCE contains carbohydrates.

Although SCCE has properties compatible with a role in the degradation of cohesive intercellular structures preceding desquamation, the physiologic function of the enzyme remains to be elucidated. A major question concerns how the activity of the enzyme is regulated in the viable epidermal layers (where it must be assumed to be synthesized and possibly stored), as well as in the stratum corneum. It will also be of interest to elucidate whether SCCE may play a role in epidermal inflammation. As suggested [15], proteolytical enzymes present in the stratum corneum intercellular space under certain conditions may be able to convert inactive forms of cytokines to active forms. Of particular interest in this context, is the biologically inactive pro-interleukin-1β, which has been shown to be produced by keratinocytes [16]. So far no epidermal enzymes have been found with the ability to convert pro-interleukin-1β to active interleukin-1β. However, because chymotrypsin and cathepsin G (a chymotrypsinlike enzyme) have the ability to catalyze the conversion of inactive 31 kD recombinant pro-interleukin-1β to a fully active 17-kD form [16–18], it is possible that SCCE can also catalyze this conversion. An enzyme that

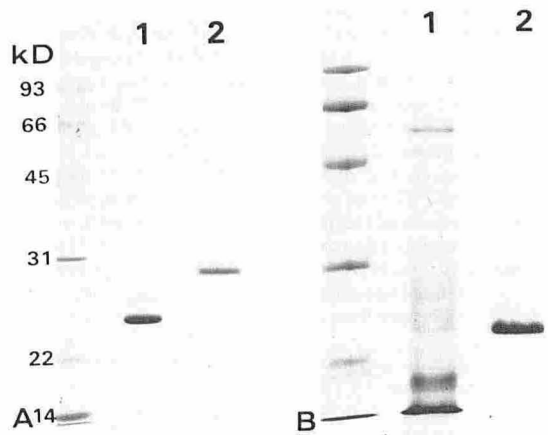

Figure 5. *A*, SDS-PAGE of SCCE purified by affinity chromatography on SBTI Affigel 15. Gel 12.5%. 1, unreduced sample; 2, reduced sample. *B*, Comparison (SDS-PAGE 12.5% gel, unreduced samples) of KCl extract of plantar corneocytes (1) and SCCE purified by affinity chromatography on SBTI Affigel 15 (2). In *column 1* the KCl extract (corresponding to *Extract 1* in Fig 1) was dialyzed against 0.1 M Tris-HCl pH 7 and concentrated 100 times by ultrafiltration before being prepared for electrophoresis by the addition of 0.1 volume of 10% SDS and 0.1 volume of glycerol. In 2, a fraction from affinity chromatography with $A_{280\ nm} = 0.14$ was concentrated three-fold before being prepared as above. Sample volumes in 1 and 2, 10 μl. *A,B,* Molecular weight markers to the left.

enzymologically appears to be identical with SCCE is present in significant amounts is psoriatic scales (Egelrud T and Lundström A; unpublished observation).

In conclusion, SCCE may have important functions in terminal epidermal turnover and in epidermal inflammation. The availability of purified SCCE makes it possible to further elucidate these functions.

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